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IMMUNOLOGY AND HOST-PARASITE INTERACTIONS - ORIGINAL PAPER

Immune response to *Neospora caninum* live tachyzoites in prepubertal female calves

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Abstract

The aim of the present study was to characterize the specific immune response in prepubertal female calves inoculated with *Neospora caninum*. Forty-eight *N. caninum*-seronegative 6-month-old Angus female calves were randomly allocated into two groups: group A calves were inoculated subcutaneously (sc) with 1×10^6 tachyzoites of the low virulence NC-Argentina LP1 isolate in sterile phosphate-buffered saline (PBS); group B calves were mock inoculated sc with sterile PBS. Calves from group A developed a specific immune response characterized by the production of IgG antibodies and the expression of IFN-γ and TNF-α cytokines. Animals did not present any febrile reaction or reactions at the site of inoculation. Although chronic *N. caninum* infection was developed in 50% of calves of group A after inoculation, according to the presence of antibodies against rNc-SAG4, antigen characteristic of bradyzoites, *N. caninum* antibodies dropped below the cut-off of ELISA from day 210 post-inoculation onwards. Future trials using the same group of inoculated animals will allow the characterization of the evolution of the immune response during pregnancy and to determine whether the immunization with the local isolate is able to prevent congenital transmission and to protect against heterologous challenges.

Keywords Prepubertal female calves · *Neospora caninum* · Live inoculation · Immune response

Introduction

Neospora caninum is an obligate intracellular protozoan that causes abortions in cattle and severe encephalomyelitis in dogs (Dubey and Schares 2011). Cattle can be infected horizontally by the ingestion of *N. caninum* oocysts shed in the feces of definitive hosts (canids) or vertically from the mother to the fetus during gestation (Dubey et al. 2007). More than one billion dollars per year are lost due to neosporosis in the cattle industry worldwide (Reichel et al. 2013), but there are not vaccines or drugs; control measures are limited to herd management (Dubey et al. 2007).

Pathophysiology on bovine neosporosis has been largely studied (reviewed in Innes et al. 2002); but the mechanisms involved in the abortion seem to be a complex relationship between the host and the parasite (Dubey et al. 2007). T-helper 1 (Th1) immune response involving the production of different cytokines including interferon-gamma (IFN-γ), interleukin-12 (IL-12), and tumor necrosis factor alpha (TNF-α) with the production of immunoglobulin G_2 (IgG_2) plays an important role in reducing the multiplication of *N. caninum* within the host (Staska et al. 2003; Almería et al. 2009).

Experimental and epidemiological data indicates that a protective immune response could be naturally developed in cattle exposed to *N. caninum* (Dubey et al. 2007). Some authors have hypothesized that early exposure of young cattle to

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N. caninum may be desirable in order to develop immunity against postnatal transmission, but only three studies have been performed in male calves (De Marez et al. 1999; Maley et al. 2001; Klevar et al. 2007) and just one in female calves (Lundén et al. 1998). Recently, Sharma et al. (2018) suggested that neonates are more resistant to cellular invasion with *N. caninum* because they had a significant greater percentage of $CD14^+$ monocytes with higher CD80 cell surface expression and greater secretion of IL-1β. The aim of the present study was to characterize the specific immune response induced in prepubertal female calves subcutaneously inoculated with *N. caninum* tachyzoites.

Materials and methods

Animals and experimental samplings

Forty-eight 6-month-old Angus female calves, in good nutritional condition and grazing on pasture, were involved in the experiment. Calves were *N. caninum*- and *Toxoplasma gondii*-seronegative by an indirect fluorescent antibody test (IFAT) (cut-off titer ≤ 1:25) (Venturini et al. 1999). In addition, calves were seronegative to bovine viral diarrhea and bovine herpesvirus by serum neutralization tests. The herd was also free of brucellosis and tuberculosis and a vaccination program against foot and mouth disease was routinely performed. Routine clinical examination and vaccination with inactivated vaccines against respiratory diseases and bovine keratoconjunctivitis (Providean Respi 8 Querato®, Tecnovax, Argentina) were performed 2 months before the start of trial.

Calves were randomly allocated into two groups. The immunogen inoculation route was the subcutaneous because it has been suggested that it may model the natural infection better than the intravenous route of inoculation (Maley et al. 2001; Benavides et al. 2014). Similarly, the dose of inoculation was chosen based on the experimental design in a calf model previously used by Maley et al. (2001). Calves from group A ($n = 24$) were inoculated subcutaneously (sc) in the neck region with 1×10^6 live tachyzoites of NC-Argentina LP1 isolate in 2 ml of sterile phosphate-buffered saline (PBS) (pH 7.2) at day 0. Calves from group B ($n = 24$) were mock sc-inoculated in the neck with 2 ml of sterile PBS. Calves were observed daily throughout the experiment. Rectal temperature was recorded for five consecutive days post-inoculation (PI) because the weather conditions were adverse on days 6 and 7 PI and it was not possible to record the temperature in the inoculated animals during these days. Animals with temperatures above 39.5 °C were considered febrile. Local inflammatory reactions at the injection site were evaluated daily for 1-week PI. Calves used in this study were handled in strict accordance with good animal practice and the conditions were approved by the Animal Ethics Committee at INTA Balcarce (CICUAE#003/2015).

NC-Argentina LP1 isolate and *N. caninum* inoculum

Live tachyzoites of the low virulence NC-Argentina LP1 isolate of *N. caninum* (Campero et al. 2015a, Campero 2017) were used to inoculate calves from group A, as well as to produce antigen for serological assays and stimulation of whole blood for cytokine production. The isolate was maintained by passage (approximately 66 passages) in Vero cells and was harvested when 80% of the parasitophorous vacuoles were undisrupted.

NC-Argentina LP-1 tachyzoites were washed three times in PBS (pH 7.4), separated from host cell debris by sequential passage through 21, 23, 25, and 27 gauge needles and filtered through Sephadex columns (Sephadex™ G-25 Medium, GE Healthcare, Sweden). Tachyzoite numbers and viability were determined by trypan blue exclusion followed by counting two aliquots of the resulting filtrate using a hemocytometer. Inoculum was transported in an insulated box at room temperature (RT) for animal inoculation within 45 min of purification from host cells. At the end of the field inoculation, an aliquot of the inoculum was re-inoculated in Vero cells to check for viability in field conditions.

Samples of female calves

Blood samples (40 ml), with or without heparin, were collected through the jugular vein. Blood samples with heparin were immediately processed to obtain plasma and peripheral blood mononuclear cells (PBMC) and for further analyses (see below). Blood samples without heparin were centrifuged at 1600×*g* for 10 min and serum stored at − 20 °C. Cellular immune response assays were performed on PBMC at days 0, 11, 15, 21, and 30 PI. Serological assays were performed on serum samples collected at days 0, 15, 21, 30, 60, 90, 120, 210, 270, 300, and 365 PI.

Native antigen extract

Native antigen extract was obtained as previously described by Innes et al. (1995). Briefly, 1×10^9 NC-Argentina LP-1 tachyzoites were purified using Sephadex columns (GE Healthcare), re-suspended in 1 ml of 10 mM Tris hydrochloride pH 7.0 containing 2 mM of phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO, USA), and disrupted by sonication (Sonifier 450, Branson Ultrasonic Co., USA). Protein content was determined using the Micro

BCA protein assay method (Pierce, Rockford, USA) and the supernatant aliquoted and cryopreserved at − 80 °C.

Specific IgG and its subisotypes in serum samples

N. caninum-specific IgG levels were determined by iELISA on serum samples as previously described by Hecker et al. (2013). Briefly, 96-well plates were coated with native antigen extract (1 μg in 100 μl/well) and bovine serum samples were diluted (1:100) in PBS/0.75 M EDTA/EGTA (pH 6.3) plus 4% skim milk. Anti-bovine IgG polyclonal antibody conjugated to peroxidase (diluted 1:1000; Sigma Chemical Co) was used as secondary antibody. *N. caninum*-specific IgG levels was evaluated using an Epoch micro-volume spectrophotometer system (Epoc, Bioteck® instruments, Inc., Vermont, USA) at optical density of 405 nm (OD_{405}) when the *N. caninum* high-positive control reached 1.0 ± 25% OD value. Serum samples were analyzed in duplicate and the mean value of the OD was converted into a relative index percent (RIPC) using the following formula: RIPC = (OD_{405} sample − OD_{405} negative control) / (OD_{405} positive control − OD_{405} negative control) × 100. A RIPC value ≥ 8.2 indicated a positive result (Hecker et al. 2013). In order to compare the results obtained by our iELISA in house with a commercial iELISA kit, serum samples from calves of group A collected at days 300 and 365 PI were tested by a commercial *N. caninum* ELISA kit (CIVTEST® BOVIS Neospora, HiPRA, Girona, Spain).

N. caninum-specific subisotypes IgG_1 and IgG_2 were determined by iELISA on serum samples collected at days 0, 11, 21, 30, and 60 PI, as previously described by Moore et al. (2011). Anti-bovine IgG1 or IgG2 monoclonal antibodies (mAbs) were used (1:100; SerotecTM, Oxford, UK). For IgG_1 and IgG_2, a kinetic reading was determined at an OD_{405} when the *N. caninum* high-positive control with anti-IgG_1 and anti-IgG_2 reached 1.0 ± 25%. Data were expressed as ratio of OD values for IgG_1/IgG_2.

Immunoblot analysis

In the inoculated animals from group A that were seronegative to the iELISA, immunoblots (IB) were performed from native antigen extract and rNc-SAG4 bradyzoite antigen (Fernandez-García et al. 2006) with serum samples as previously described by Campero et al. (2015b) and Aguado-Martínez et al. (2008), respectively. The reaction against five immunodominant antigens (IDAs) with relative molecular masses of 19, 29, 30, 33, 37 kDa was recorded in the IB from crude tachyzoite antigen and a sample was considered positive when two or more IDAs were recognized (Schares et al. 1999).

Phenotypic analysis of PBMC using flow cytometry

Percentages of T cell subsets ($CD4^+$, $CD8^+$, and $\gamma\delta^+$) were analyzed in PBMC from blood samples collected at days 0, 11, 15, and 21 PI by flow cytometry after immune-labeling with mAbs specific for bovine leucocyte subpopulations: anti-CD4 (IgG_{2a}, CC8 clon), anti-CD8 (IgG_{2a}, CC63 clon), and anti-WC1 to differentiate γδ T cell populations (IgG_{2a}, CC15 clon) (AbD Serotec, Raleigh, North Carolina, EE.UU). As secondary antibodies, rat anti-bovine IgG isotypes conjugated with FITC or PE (AbD Serotec) were used.

Cell suspensions (5×10^5 cells/well) were incubated with mAbs diluted in PBS at 4 °C for 20 min. Cells were then washed with PBS, re-suspended in 100 μl of fluorescence-activated cell sorting (FACS) buffer flow (Becton, Dickinson and Company, Franklin Lakes, USA) and fixed with 0.05% formaldehyde. Isotype controls were used as negative controls. Analysis was performed using a FAC Scan cytometer and Cell Quest software (Becton, Dickinson and Company). Ten thousand events were analyzed and the results expressed as the percentage of positively immunolabeled cells.

Cytokine mRNA expression in PBMC

Total RNA was extracted from 1×10^6 cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. RNA was digested with DNase I Amplification Grade (Invitrogen, Carlsbad, CA, USA) during 30 min at 37 °C to remove any contaminating genomic DNA (gDNA). The quality and quantity of the resulting RNA were determined using an Epoch Microplate Spectrophotometer (BioTeK, Winooski, VT, USA). All RNA samples were stored at − 80 °C until cDNA synthesis was performed.

Synthesis and amplification of cDNA were performed by PCR according to González Altamiranda et al. (2013) with minor modifications. Briefly, for cDNA synthesis, 10 μl of RNA, 1 μl (0.02 μg/μl) of random hexamers (Biodynamics), and 5 μl of dH_2O were denatured at 65 °C during 5 min and cooled on ice. Each reaction tube was completed with 5-μl RT buffer (0.05 mM potassium phosphate pH 7.2, 0.01 mM DTT 0.2% Triton X-100, and 10% glycerol), 1 μl (5 mM) dNTP (Biodynamics), 1 μl $MgCl_2$ (25 mM), 0.5 μl RNA Guard (Promega), 0.5 μl (6 U) Moloney murine leukemia virus reverse transcriptase (Promega), and 1 μl dH_2O to give a final reaction volume of 25 μl, and incubated for 90 min at 37 °C.

Real-time PCR was performed using primers for bovine interferon-γ (IFN-γ), interleukin-12 p40, tumor necrosis factor-α (TNF-α), interleukin-4 (IL-4), interleukin-10 (IL-10), and the housekeeping gene β-actin on cDNA samples from PBMC collected at days 0, 11, 15, and 21 PI, as previously reported (Regidor-Cerrillo et al. 2014). Relative

quantification of cytokine mRNA expression levels was carried out using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Cytokine protein assays

Cytokine protein levels were evaluated from PBMC collected at days 0, 15, 21, and 30 PI stimulated with *N. caninum* antigen. PBMC were obtained by centrifugation of blood in a Ficoll-Paque™ plus gradient (GE Healthcare). Cell suspensions (1×10^6 cells/well) were cultured in duplicate wells in 96-well tissue culture plates (Cellstar Greiner, Monroe, USA) with 0.1 ml of PBS (unstimulated control) or pokeweed (Pokeweed mitogen, Sigma) at 10 μg/ml to assess their ability to respond to stimulation and secrete cytokines, or with native antigen extract from the NC-Argentina LP1 strain (10 μg/ml). Plates were incubated for 48 h at 37 °C in 5% CO_2 atmosphere. Culture supernatants were collected, and IFN-γ, IL-12 p40, IL-10, IL-4, and TNF-α levels were measured by ELISA. IFN-γ level was measured as previously described by Quattrocchi et al. (2014). Briefly, Immulon II plates were coated with mAb IFN-γ (kindly donated by Dr. Babiuk) in carbonate–bicarbonate buffer, pH 9.6. Plates were blocked with PBST–0.1% BSA. Dilutions of samples and recombinant IFN-γ standard (Serotec, UK) were added. Plates were washed and rabbit polyclonal anti-IFN-γ antibody was added. After incubation, plates were washed and biotin-conjugated antibody anti-rabbit IgG was added. After incubation, alkaline phosphatase-conjugated streptavidine (KPL, USA) was added. Plates were washed, incubated with p-nitrophenylphosphate as substrate, and read at 405 nm. IFN-γ concentration was calculated from a standard curve obtained with serial dilutions of a recombinant bovine IFN-γ (Serotec). The initial concentration of the standard IFN-γ was 50,000 picogram/ml (pg/ml).

Interleukin-10 and IL-12 p40 levels were measured using a sandwich ELISA. Briefly, Immulon II plates were coated with capture mAb IL-10 (MCA 2110, ABD Serotec, Oxford, UK) and capture mAb IL-12 p40 (MCA 1782EL, ABD Serotec, UK), respectively, in carbonate–bicarbonate buffer, pH 9.6. Plates were blocked with PBST–0.1% BSA. Dilutions of samples were added. Plates were washed and a biotin-conjugated detector anti-IL-10 (MCA 2111B, ABD Serotec, Oxford, UK) and a biotin-conjugated detector anti-IL-12 p40 antibodies were added, respectively. After incubation, alkaline phosphatase-conjugated streptavidine (KPL, USA) was added. Plates were washed, incubated with p-nitrophenylphosphate as substrate, and read at 405 nm. Because recombinant standards for IL-10 and IL-12 were not available, only the OD variations through time were supplied.

IL-4 and TNF-α levels were measured in supernatants using Bovine Screening Set kits (Thermo-Fisher Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Color reaction was developed by the addition of 3,3′,5,5′-tetramethylbenzidine substrate, and reactions were stopped by adding 2 N H_2SO_4 according to the manufacturer's instructions. Plates were read at 450 nm. The cytokine concentrations were calculated from a standard curve generated with recombinant cytokines provided with the kits. The concentrations of IFN-γ, TNF-α, and IL-4 were expressed in pg/ml.

Statistical analysis

Analysis RIPC values from serum antibody responses, percentages of T cell subsets (CD4+, CD8+ and γδ+), and cytokine protein level production were compared between groups by using PROC-MIXED SAS for one-way repeated measures analysis of variance (ANOVA) with treatment as the grouping factor and time as the repeated measures factor (Littell et al. 1998). Analysis non-parametric two-tailed Mann–Whitney tests and Kruskal–Wallis test followed by a Dunn's multiple range test for all pair-wise analyses were conducted on the pooled data in order to investigate statistically significant differences in the cytokine RNA levels of PBMC among animals from different groups using GraphPad Prism 5 v.5.01 (San Diego, CA, USA). Statistical significance was reached when $P \leq 0.05$.

Results

Neither apparent clinical signs nor significant local reactions were observed in any calves inoculated with live parasites (group A) or PBS (group B) throughout the study. Group A or group B female calves did not develop fever from 24 to 120 h PI either (data not shown). The viability of the inoculum used in the field was confirmed because *N. caninum* parasites were observed in Vero cell cultures 48 h after reinoculation.

Neospora-specific *IgG production*

RIPC values are shown in Fig. 1. *Neospora*-specific IgG antibodies were significantly higher in the serum from animals from group A as compared with similar samples from calves from group B. This difference was observed at day 21 ($P < 0.001$) until day 120 PI ($P < 0.05$). The highest antibody titer in group A was detected at day 60 PI ($P < 0.001$). No significant differences between both groups were observed from day 210 until 365 PI ($P > 0.05$).

In addition, serum samples collected on day 300 and 365 PI from calves of group A were tested with a commercial *N. caninum* iELISA with a good agreement between both

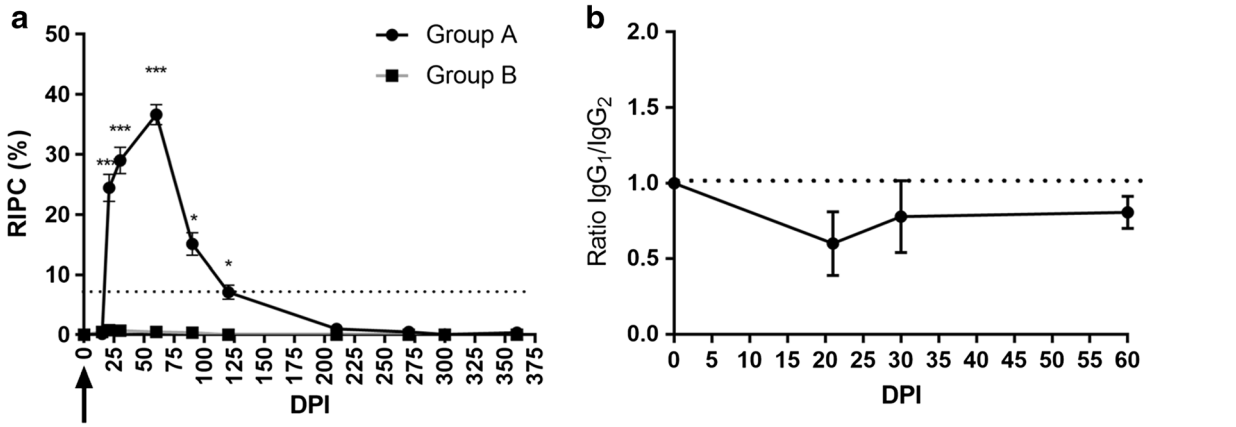

Fig. 1 Longitudinal profile of immunoglobulin G (IgG) and subisotypes in female calves of group A (inoculated with live tachyzoites of NC-Argentina LP1) and group B (mock-inoculated with PBS) assessed by an indirect ELISA. Each point represents the mean ± standard deviation (SD) at the different sampling times for groups A and B. **a** Mean ± SD of the relative index percentages (RIPC) ± SD of total IgG, through 365 days post-inoculation (DPI). Cut-off: ≥ 8.2 RIPC. The significant statistical differences between groups were analyzed: $*P < 0.05$; $***P < 0.001$. Black arrow: day of inoculation. **b** Mean of the ratio of *Neospora*-specific IgG1/IgG2 antibodies ± SD of the ratio IgG_1/IgG_2 of animals of group A through 60 DPI

iELISA techniques (Cohen's kappa coefficient: 0.857). Only 1 calf on day 300 and 2 calves on day 365 PI of 24 analyzed were positive with a low antibody titer (data not shown).

Neospora-specific *IgG subisotype production ratios*

Figure 1b shows the ratio IgG_1/IgG_2 in animals from group A until day 60 PI. Group A calves showed similar levels of IgG1 than IgG2 (ratio IgG1/IgG2 = 1) at day 0 PI. However, after day 21 PI, when the specific IgG responses were detected, the levels of IgG2 were higher than IgG1 (ratio IgG1/IgG2 < 1).

Immunoblot analysis

As the animals of group A were negative to iELISA from day 120 PI, immunoblot analysis (IB) was performed with serum samples from group A collected at 120, 210, 270, and 365 days PI. Results of specific IgG against a *N. caninum* native antigen extract, detected by IB, are presented in Table 1. At day 120 PI, 19/22 group A calves were positive to IB. The number of positive calves decreased, and on day 365 PI, only 8/21 calves from group A were positive. Only two calves of group A were negative from day 120 until 365 PI (#12 and #13) and from days 210 until 365 PI (#11 and #20). Antibodies against rNC-SAG4 antigen were detected in serum samples of 50% of calves of group A evaluated until day 365 PI (Table 1). Animals that were positive to IB against recombinant Nc-Sag4 in at least one sampling were considered chronically infected. Three animals were positive in all evaluated samplings by both IB and 19 calves showed fluctuating levels of *N. caninum*-specific IgG. The group B calves remained negative to IB throughout the assay.

Phenotypic analysis of PBMC using flow cytometry

Percentages of $CD4^+$, $CD8^+$, γδ $WC1^+$ T cell subsets from group A and B calves are shown in Fig. 2. The most remarkable change in PBMC subpopulations was a transient decrease in percentages of $CD4^+$ cells (Fig. 2a) and $\gamma\delta^+$ T cells (Fig. 2b) in female calves from group A in comparison with calves of group B at day 15 PI ($P > 0.001$). No variation of $CD8^+$ cell percentage was detected between both groups or within each group over the time ($P > 0.05$) (Fig. 2c).

Cytokine mRNA expression

The relative levels of cytokine mRNA in PBMC from group A is shown in Fig. 3. IFN-γ and TNF- α mRNA levels in group A were significantly higher at day 21 PI than pre-infection (day 0 PI), 1.51-fold change ($P < 0.05$), and 19.88-fold change ($P < 0.001$), respectively (Fig. 3a, b).

IL-12 p40 mRNA levels (Fig. 3c) in PBMC of group A were only significantly higher than pre-infection at day 11 PI (2.42-fold change) ($P < 0.05$). IL-10 mRNA levels in PBMC of group A did not change through time ($P > 0.05$). There were no changes in expression of IFN-γ, TNF- α, IL-12 p40, or IL-10 mRNA levels in PBMC from group B through the time. IL-4 mRNA was only detected in less than 50% of the PBMC samples from groups A and B and was not evaluated.

Cytokine protein assays

Significant differences in IFN-γ and TNF-α protein levels in cultured PBMC supernatant from group A were observed through time ($P < 0.001$). A significant increase in IFN-γ levels in cultured PBMC supernatant from group A was

Table 1 Specific IgG against a *Neospora caninum* native antigen extract and specific IgG against *N. caninum* recombinant SAG4 (Nc-SAG4), detected by immunoblot (IB), using polyclonal sera from female calves inoculated with the NC-Argentina LP1 isolate (group A). Serum samples were collected 120, 210, 270, and 365 days post-inoculation (DPI)

Animals #	IB against a *N. caninum* native antigen extract				IB against a recombinant Nc-SAG4
	Day 120 PI	Day 210 PI	Day 270 PI	Day 365 PI	Animals that were positive at least in one sampling
1	S/D[a]	S/D[a]	S/D[a]	S/D[a]	S/D[a]
2	+	+	+	+	+
3	+	−	+	−	+
4	+	+	+	−	−
5	+	+	+	−	−
6	+	−	+	+	+
7	+	S/D[a]	S/D[a]	S/D[a]	S/D[a]
8	+	+	+	−	−
9	+	S/D[a]	−	+	−
10	+	+	−	+	+
11	+	−	−	−	+
12	−	−	−	−	+
13	−	−	−	−	+
14	+	+	+	+	−
15	−	+	−	−	−
16	+	−	S/D[a]	S/D[a]	S/D[a]
17	+	+	−	−	+
18	S/D[a]	S/D[a]	−	−	−
19	+	+	+	+	+
20	+	−	−	−	−
21	+	+	+	+	+
22	+	−	S/D[a]	−	−
23	+	+	S/D[a]	−	S/D[a]
24	+	+	+	+	−

[a] S/D: the sera of these animals were not available

detected from day 15 until day 30 PI ($P < 0.001$) in comparison with levels observed in group B (Fig. 4a). A high TNF-α level was observed at day 15 and 21 PI in group A compared to group B ($P < 0.001$) although these differences between both groups were not evident at day 30 PI (Fig. 4b). There were no significant differences in IL-4, IL-10, and IL-12 p40 levels in PBMC culture supernatant between groups A and B and throughout the experiment (Fig. 4c, d, e).

Discussion

A promising approach for the development of a live vaccine against neosporosis is the use of low-virulent *N. caninum* isolates. Interestingly, isolates of *N. caninum* from congenitally infected asymptomatic calves have shown limited capacity to cause abortion in cattle and therefore could be good vaccine candidates (Regidor-Cerrillo et al. 2008). The NC-Nowra and NC-Spain1H isolates, both isolated from asymptomatic calves, have been inoculated in cows before mating and showed promising protection against vertical transmission depending on the type of inoculum formulation and the administration route (Williams et al. 2000, 2007; Rojo-Montejo et al. 2009, 2013; Weber et al. 2013). Preliminary in vitro studies carried out with NC-Argentina LP1 isolate (Campero et al. 2015a), obtained from a congenitally infected asymptomatic calf, showed a lower invasion rate than the NC-Spain7 isolate, evidence of lower virulence than the reference isolate (Regidor-Cerrillo et al. 2011; Campero 2017). In the present study, the inoculation with live tachyzoites of NC-Argentina LP1 isolate in 6-month-old female calves generated specific cellular immune responses with specific antibody levels that decreased at day 120 PI.

It has been suggested that although the subcutaneous inoculation is an artificial route of inoculation of tachyzoites, the natural infection could be a model better than the intravenous route (Benavides et al. 2014). Other authors mentioned that the immune response of cattle sc infected appears to correlate

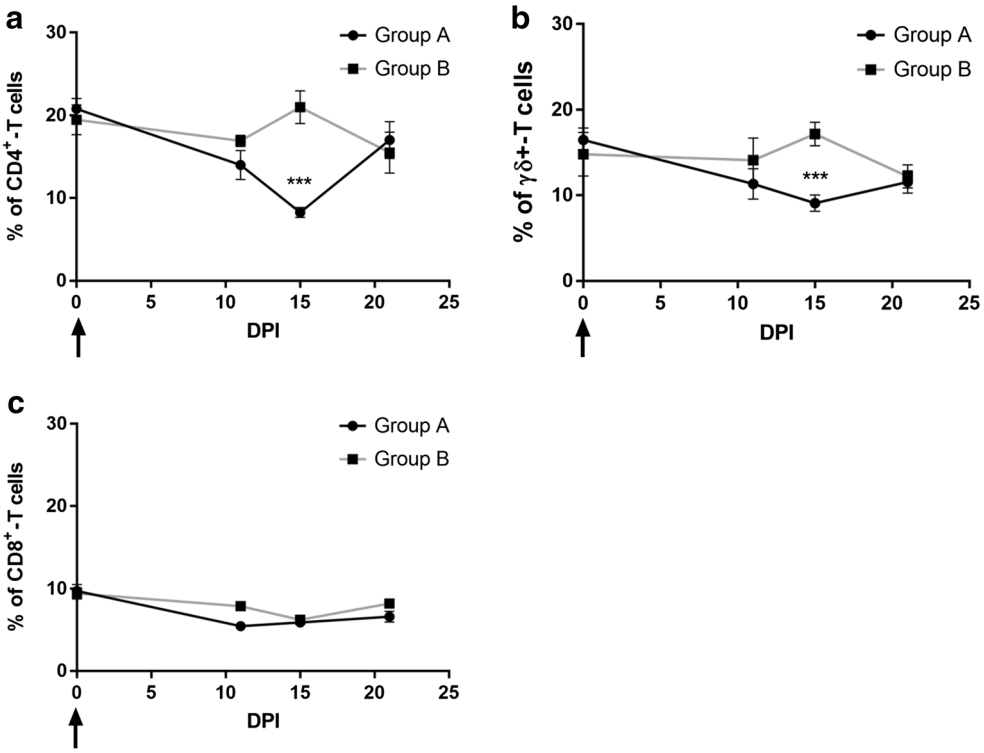

Fig. 2 Variation of the percentage (%) of T cells in female calves of group A (inoculated with live tachyzoites of NC-Argentina LP1) and group B (mock-inoculated with PBS), measured by flow cytometry at days 0, 11, 15, and 21 post-inoculation. Each point represents the mean ± SD at the different sampling times for groups A and B. **a** $CD4^+$, **b** $\gamma\delta^+$, and **c** $CD8^+$. Black arrow: day of inoculation. DPI: days post-inoculation. The significant statistical differences between groups were analyzed: $^*P < 0.05$; $^{***}P < 0.001$: $^*P < 0.05$; $^{***}P < 0.001$

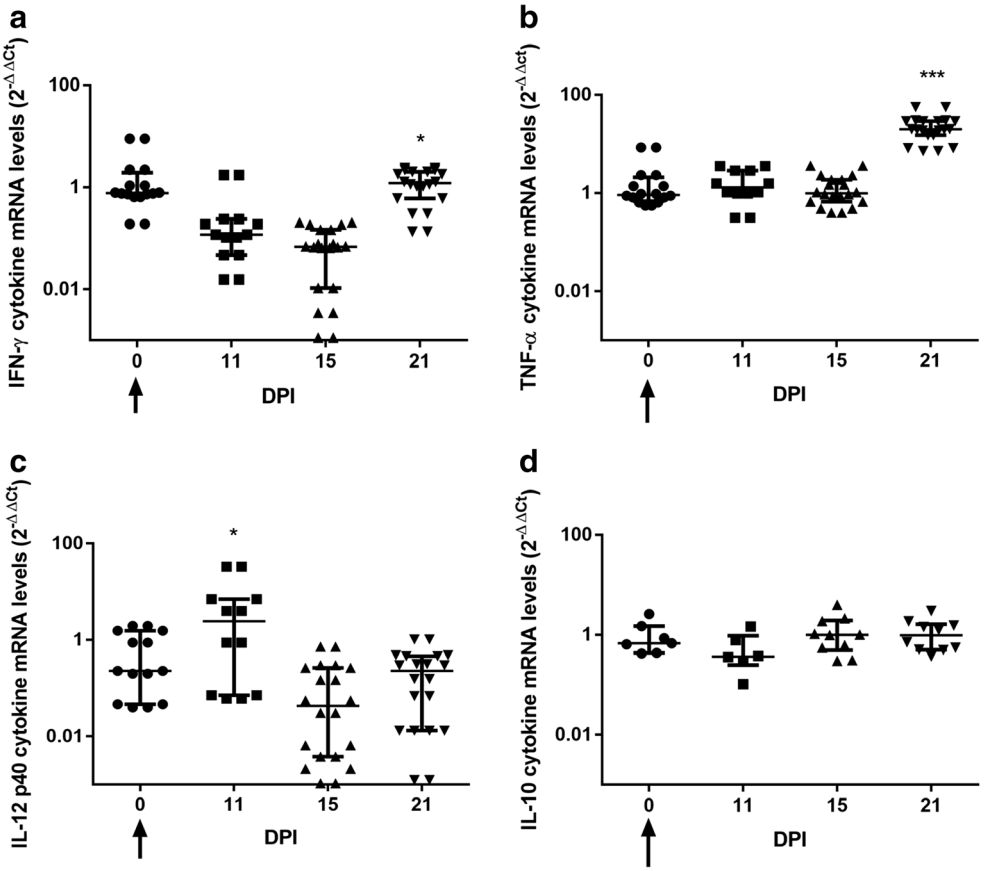

Fig. 3 Scatter-plot graphs of relative cytokine mRNA expression levels in PBMC from in female calves of group A (inoculated with live tachyzoites of NC-Argentina LP1). Relative quantification of cytokine mRNA expression levels was carried out using the $2^{-\lambda\lambda Ct}$ method. **a** INF-γ, **b** TNF-α, **c** IL-12 p40, and **d** IL-10. Horizontal lines represent median values for each group. Black arrow: day of inoculation. DPI: days post-inoculation. The significant statistical differences through time in group A were analyzed: $^*P < 0.05$; $^{***}P < 0.001$

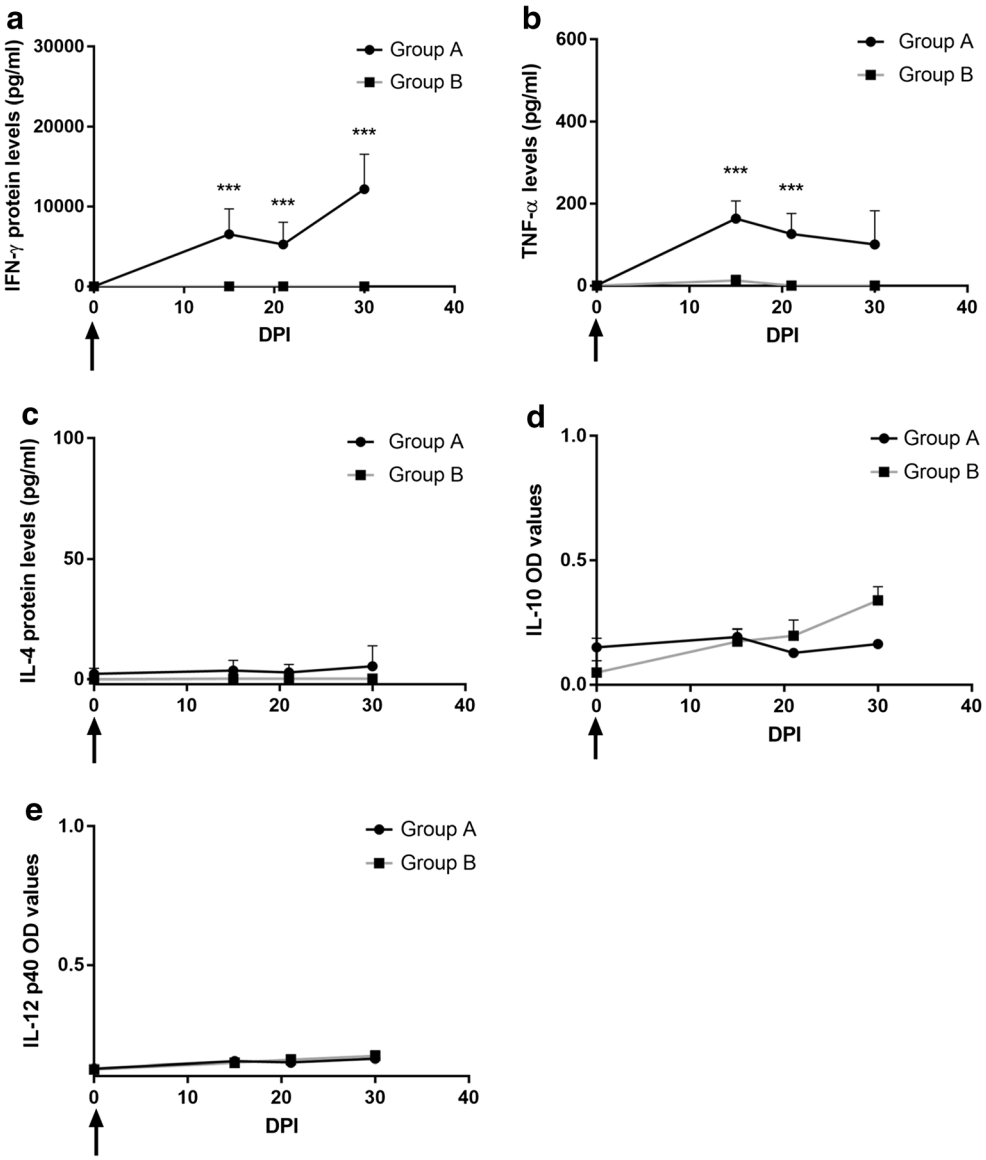

Fig. 4 Cytokine protein levels in culture supernatants of stimulated PBMC from female calves of group A (inoculated with live tachyzoites of NC-Argentina LP1) and group B (mock-inoculated with PBS). Each point represents the mean ± SD at the different sampling times for groups A and B. The concentrations of **a** IFN-γ, **b** TNF-α, and **c** IL-4 are expressed in picogram/ml (pg/ml). For **d** IL-10 and **e** IL-12 p40, only the OD variations through time were displayed. Black arrow: day of inoculation. DPI: days post-inoculation. The significant statistical differences between groups were analyzed: $*P < 0.05$; $***P < 0.001$

well with that of animals infected orally (Maley et al. 2001). In agreement with results previously reported by Maley et al. (2001), using a low dose (5×10^6) of *N. caninum* tachyzoites of NC1 isolate, in the present study, the subcutaneous inoculation has shown to be safe because the animals did not show febrile reaction or reactions at the inoculation site.

Other researchers have found different results with other ELISAs, particularly for low antibody titers (Wouda et al. 1998; Atkinson et al. 2000) but in the present study, when the serum samples from calves of group A were analyzed by a commercial iELISA kit, a good agreement was obtained between both iELISA techniques. The results of the present study showed that *N. caninum*-specific antibody levels were undetectable from day 210 PI until day 365 PI. This is likely because the levels of the *Neospora*-specific antibody were below the cut-off limit detected by the soluble extract-based ELISA and commercial *N. caninum* ELISA. However, IB results showed that almost all inoculated calves were positive for the NC-Argentina LP-1 isolate at least in one sampling. Only 1 out of 24 calves was negative from day 210 to day 365 PI both for total IgG and for anti-rNc-SAG4. Previously, fluctuations in antibody levels were described in calves experimentally inoculated with *Neospora* up to 1 year after the initial sc infection (Maley et al. 2001). Okeoma et al. (2004) stated that the pattern of *N. caninum* tachyzoite antigen recognition by IB in sera from naturally infected cows correlated with the level of seropositivity of the serum sample at the time point. Possibly, the detection failure by IB in some samplings in the present study may be due to the fluctuation of low remaining antibody over time.

All female calves inoculated also developed *Neospora*-specific IgG_1 and IgG_2 from day 15 PI as described previously (Uggla et al. 1998; Lundén et al. 1998; Maley et al. 2001; Klevar et al. 2007). In

agreement with our results, De Marez et al. (1999) described that 2.5-month-old calves orally infected with *N. caninum* oocysts developed *Neospora*-specific IgG_1 and IgG_2 antibodies between 2- and 4-week PI.

In the present study, no variation in the $CD8^+$ T cell percentage was observed associated with *N. caninum* infection over time. However, a transient decrease at day 15 PI followed by an increase at day 21 PI in percentages of $CD4^+$ T cells was observed in inoculated cattle. In relation to these results, we previously reported the ratio of $CD4^+/CD8^+$ in pregnant cattle versus fetal lesion score (Hecker et al. 2013) and we observed that animals receiving live tachyzoites prior to gestation had fewer fetal lesions and a high ratio $CD4^+/CD8^+$ compared to those animals vaccinated with native antigen extract plus ISCOMs. Studies investigating the mammalian immune response to infection with *N. caninum*, primarily performed in mice and then in bovine, indicated that $CD4^+$ but not $CD8^+$ T lymphocytes have an important role for protection against *N. caninum* infection (Tanaka et al. 2000; Staska et al. 2003).

Our results showed a decrease in the percentages of $\gamma\delta^+$ T cells after *N. caninum* live tachyzoites infection. Hecker et al. (2013) did not mention changes in the percentages of $\gamma\delta^+$ T cells in heifers infected with live tachyzoites of NC-6 Argentina isolate. Possibly in our work, we could see variations in the $\gamma\delta^+$ T cells because this T cell population constitute up to 50% of all T cell population in the peripheral blood and lymphoid organs of young cattle (Hein and Mackay 1991). The $\gamma\delta^+$ T cells respond to antigens and might play a key role in *Neospora* infections (Hemphill et al. 2016). Klevar et al. (2007) mentioned a decrease in the percentage of circulating NK cells at days 4–6 PI in calves infected with *N. caninum.* This happens possibly because of cellular migration within affected tissues stimulated by cytokines and chemokines, produced by activated NK cells. We can hypothesize that the transitory decrease of $CD4^+$ and $\gamma\delta^+$ T cells in the PBMC could be associated with the activation of immune responses and immune cell migration to lymph nodes to initiate the adaptive immune response.

Previous works described that the protective immune response against *N. caninum* was dominated by an increase in the production of Th1 cytokines like IFN-γ and TNF-α and a decrease of cytokines like IL-4 (Khan et al. 1997; Baszler et al. 1999; Innes et al. 2002; Rosbottom et al. 2008; Regidor-Cerrillo et al. 2014). In the present study, higher levels of IFN-γ and TNF-α (mRNA levels and protein in stimulated PBMCs) were detected in inoculated calves from day 15 PI indicating that the infection with *N. caninum* promoted TH1 immune responses characterized by pro-inflammatory cytokines linked to a protective immune response against the protozoa.

According to this pro-inflammatory status, although IL-12 production was not detected after PBMC stimulation, the IL-12 p40 mRNA levels were significantly higher prior to the increase of IFN-γ and TNF-α mRNA expression at day 11 PI in circulating PBMCs of infected calves. Early IL-12 production during *N. caninum* infection has been associated with development of innate immunity (Behzadi et al. 2016). Furthermore, other authors have reported that IL-12 facilitates Th1 responses by stimulating the differentiation of Th1 cells and IFN-γ secretion by antigen-activated Th1 cells (Magram et al. 1996; Behzadi et al. 2016). Moreover, it has been mentioned that IL-12 inhibited IL-10 and IL-4 secretion (Behzadi et al. 2016). In agreement with these findings, neither IL-10 and IL-4 RNA expression nor their protein levels after PBMC stimulation were detected in infected calves in the present work. IL-10 is a regulatory cytokine that is triggered when the Th1 response is exacerbated and, in this way, can counter-regulate inflammatory responses (Bancherau et al. 2012). In this study, it is likely that the prepubertal inoculation of calves induced a controlled cellular immune response and a non-detectable IL-10 level. Recently, Sharma et al. (2018) suggested that neonates are more resistant to cellular invasion with *N. caninum*.

Previous reports mentioned that endogenous transplacental transmission is more likely to occur in cattle that were infected congenitally than in those that were infected postnatally, probably due to the development of incomplete immunity when the infection occurs during gestation (Horcajo et al. 2016; Marugán-Hernández 2017). Nevertheless, it is unknown if a cow that was experimentally infected with *N. caninum* prior its puberty will transmit the parasite during its gestation. Based on the findings of this study, our research group will continue with future trials using the same group of inoculated animals that will allow us to characterize the evolution of the immune response during pregnancy and to determine if the immunization with the local isolate is able to prevent congenital transmission and to protect against heterologous challenges.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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